Detection of Aneuploidy in Human Spermatozoa of Normal Semen Donors by Fluorescence *in Situ* Hybridization

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We have studied human spermatozoa from 24 normal, healthy unexposed men, 18 of whom were semen donors at the Sperm Bank in Turku, using multicolor fluorescence *in situ* hybridization with two chromosome-specific probes. The possible age-related increase in aneuploidy frequencies was assessed. Ten thousand spermatozoa were scored per individual for the presence of hyperploid, i.e., disomic and diploid, cells. The overall hybridization efficiency was 98.8%. The frequency of spermatozoa with two chromosome 1 signals was 11.5 ± 5.2/10,000. The frequency of spermatozoa with two chromosome 7 signals was 6.4 ± 3.9/10,000. Diploidy was present in 15.0 ± 8.9/10,000 spermatozoa. Interindividual variation was quite large. No statistically significant correlation between age of the donors (range = 20–46 years) and the frequency of hyperploid spermatozoa was observed. The results give background information on the incidence of hyperploid spermatozoa in unexposed men and encourage the use of this novel technique for future studies on genetic effects in men exposed to potentially aneuploidogenic agents. — Environ Health Perspect 104(Suppl 3):629–632 (1996)

Key words: aneuploidy, chromosome 1, chromosome 7, fluorescence *in situ* hybridization, hyperploidy, semen , spermatozoa

Introduction

Germ cell chromosome aberrations, either numerical or structural, may cause pregnancy delay, spontaneous abortions, fetal and perinatal mortality, or severe malformation syndromes in newborns. Numerical chromosome errors may arise at maternal or paternal meiotic divisions or at first cleavages of the zygote. Although aneuploidy is more common in oocytes than in spermatozoa, it is in practice much easier to study spermatozoa and to obtain statistically meaningful cell numbers. Direct analysis of chromosome numbers in human spermatozoa has gained large interest since the invention of chromosome-specific fluorescence *in situ* hybridization (FISH) techniques (*1–9*).

Human spermatozoa carry many more chromosomal abnormalities than germ cells of experimental animals (*10*). It has been suggested that one reason might be exposure of men to various environmental agents. To estimate whether environmental mutagens can affect segregation of chromosomes in male meiosis, the frequency and variation of aneuploidy in spermatozoa of normal unexposed men must first be characterized. We have studied 24 normal semen donors and analyzed the frequency of hyperploid spermatozoa by using multicolor FISH with probes for chromosomes 1 and 7. The possible effect of age on aneuploidy frequencies in spermatozoa was evaluated.

Methods

Semen Donors and Semen Analyses

Ejaculates were obtained from 18 normal healthy donors, all nonsmokers, who had fulfilled the criteria for becoming semen donors for an artificial insemination center at the Semen Laboratory of the University of Turku. In addition, six healthy medical students with normal semen analysis results, but with unknown fertility status, were studied. All donors gave written informed consent. A complete semen analysis was performed for each sample using World Health Organization criteria (*11*). Semen samples were stored at −70°C in closed plastic straws until thawed at room temperature.

Preparation of Slides and Sperm Nuclear Decondensation

Seven-microliter aliquots of semen were spread on clean microscope slides and allowed to air dry at room temperature for at least 16 hr.

Sperm nuclear decondensation was accomplished essentially as described by Robbins et al. (*6*) with slight modifications. Seminal smears were incubated in 10 mM dithiothreitol (DTT, Sigma Chemical Co., St. Louis, MO) 15 min, followed by incubation in 4 mM lithium diiodosalicylate (Sigma)/1 mM DTT for 30 min. Smears were allowed to air dry before the hybridization procedure.

Probe Generation on Fluorescence *in Situ* Hybridization

The probe for the pericentric heterochromatin of chromosome 1, pUC 1.77, was a gift from J. Wiegant, University of Leiden, The Netherlands, and was labeled with biotinylated deoxyuridine triphosphate (dUTP; Boehringer Mannheim, Mannheim, Germany) using a nick translation kit (Boehringer Mannheim) and purified using a TE Select D G-25 column (5 Prime-3 Prime, Boulder, CO).

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Abbreviations used: DTT, dithiothreitol; dTTP, deoxythymidine triphosphate; dUTP, deoxyuridine triphosphate; FISH, fluorescence *in situ* hybridization; FITC, fluorescein isothiocyanate; LRSC, lissamine rhodamine; PCR, polymerase chain reaction; SSC, saline–sodium citrate solution.

A primer set for amplification of the chromosome 7-specific alpha satellite was made with an oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). Polymerase chain reaction (PCR) was performed using digoxigenin-11-dUTP (Boehringer Mannheim) in the reaction mixture in order to label the probe while synthesizing. The PCR conditions were adopted from Dunham et al. (*12*) with the following modifications. The deoxythymidine triphosphate (dTTP) concentration was 133 µM, the digoxigenin-11-dUTP concentration was 66 µM, and the amount of *Taq* DNA polymerase (Boehringer Mannheim) was 2.5 units per reaction. Thirty cycles were run with denaturation at 92°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 30 sec. The PCR product was not purified for hybridization.

For *in situ* hybridization, 80 ng of both probes and 6.0 µg of herring sperm DNA in 60% deionized formamide/2×SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate) were used per slide in a volume of 100 µl. The target and the probe were denaturated simultaneously at 75 to 80°C for 10 min in an oven followed by hybridization at 37°C for 16 to 24 hr. Post-hybridization washes were carried out three times for 5 min each at 43°C in 60% formamide/2×SSC, pH 7.0, two times in 0.2×SSC for 5 min at room temperature, and 5 min in TN-buffer (0.1 M Tris–HCl, 0.15 M NaCl) containing 0.05% Tween 20. The slides were blocked for 20 min at 37°C using 0.5% blocking reagent (Boehringer Mannheim) in TN-buffer. For fluorescence detection of hybridization the slides were incubated 45 min with 20 µg/ml sheep antidigoxigenin (Boehringer Mannheim) at 37°C and 30 min with 10 µg/ml donkey antisheep-fluorescein isothiocyanate (FITC; Chemicon, Temecula, CA) at 37°C in blocking solution. In the latter incubation, 5 µg/ml of lissamine rhodamine (LRSC)-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) was added. As suggested by Williams et al. (*7*), propidium iodide (5 ng/ml; Sigma) was used as a weak counterstain in 2.5% DABCO antifade (Sigma) diluted in 90% glycerol–1.0 M Tris–HCl, pH 7.5. The slides were examined at 1,250× magnification with a Zeiss Axioplan fluorescence microscope equipped with a double band pass filter that allows simultaneous detection of both red and green fluorescence. Photomicrographs were taken on Agfachrome RS 100 film.

Ten thousand spermatozoa were scored by two scorers, 5,000 spermatozoa each. The following criteria were used: only signals with compact, clear appearance were taken into account. If scattered string-of-pearls-like signals were observed or in the case of poor hybridization (most spermatozoa without any signals), the whole microscopic field was excluded. The morphology of the spermatozoa had to be well maintained, and overlapping cells were excluded. For twin signals, only those cases were accepted where two compact signals were clearly separate, with a distance at least the diameter of one signal. The frequency of spermatozoa with 0, 1, or 2 red and 0, 1, or 2 green signals was determined.

The correlation analysis of age and frequencies of spermatozoa with hyperploid chromosome numbers were performed using a SAS statistical program package [SAS Institute, Cary, NC (*13*)].

Results

The hybridization efficiency of this technique was good. When spermatozoa with no signals or with just one signal (either green or red) were considered as signs of poor hybridization, the average efficiency was 98.8% (Table 1).

In the results of the analyses shown in Table 1, 98.4% of all spermatozoa showed a normal chromosome constitution with these probes, i.e., one red and one green signal. The frequency of spermatozoa lacking a chromosome 1 signal was 2.3 ± 1.7/10,000

Table 1. Analysis of FISH signals (red, chromosome 1; green, chromosome 7) in human spermatozoa of 24 normal semen donors.

		Number of sperm						
Donor	Age (years)	No signals	1 Green only	1 Red only	1 Green, 1 red[a]	1 Green, 2 red[b]	2 Green, 1 red[c]	2 Green, 2 red[d]
1	23	31	6	11	9918	5	14	15
2	31	26	0	6	9933	13	7	15
3	34	19	1	3	9948	12	12	5
4	38	376	5	2	9599	9	2	7
5	45	7	4	5	9973	4	2	5
6	40	47	1	12	9919	14	1	6
7	44	41	4	9	9929	5	3	9
8	38	296	3	9	9635	16	7	34
9	31	66	2	3	9912	4	6	7
10	43	16	1	5	9959	10	1	8
11	49	77	2	3	9864	16	9	29
12	44	23	1	8	9946	3	8	11
13	46	152	3	10	9796	11	7	21
14	29	91	1	4	9878	11	3	12
15	37	102	0	13	9823	18	14	30
16	22	63	5	4	9891	11	9	17
17	21	314	3	4	9661	9	2	7
18	20	170	1	15	9784	15	3	12
19	23	146	2	7	9803	21	8	13
20	23	157	1	13	9780	14	6	29
21	21	100	1	12	9850	17	6	14
22	21	87	4	5	9852	15	8	29
23	22	130	3	10	9814	18	11	14
24	30	168	1	3	9809	4	4	11
Mean ± SD	32.3 ± 9.8	112.7 ± 98.5	2.3 ± 1.7	7.3 ± 3.9	9845 ± 102	11.5 ± 5.2	6.4 ± 3.9	15.0 ± 8.9

Ten thousand spermatozoa were scored per donor and altogether, 240,000 spermatozoa were studied. Lack of signals was interpreted as poor hybridization; lack of one signal may also indicate loss of a chromosome (nullisomy). [a]Normal. [b]Disomy-1. [c]Disomy-7. [d]Diploidy.

and the frequency of spermatozoa lacking a chromosome 7 signal was 7.3 ± 3.9/10,000. Previous studies have suggested that nullisomy cannot be reliably scored because it can also represent lack of hybridization.

Our study focused on the incidence of hyperploid spermatozoa. The frequency of spermatozoa with two chromosome 1 signals was 11.5 ± 5.2/10,000. The frequency of spermatozoa with two chromosome 7 signals was 6.4 ± 3.9/10,000. Diploidy was found in 15.0 ± 8.9/10,000 spermatozoa.

The interindividual variation was quite large. For example, donors 8, 11, and 15 had higher frequencies of aneuploid spermatozoa than other donors (Table 1). No statistically significant correlation between the age of the donors (range = 20–46 years) and the frequency of spermatozoa with two chromosome 1 signals ($p = 0.1449$), spermatozoa with two chromosome 7 signals ($p = 0.3833$), or diploid spermatozoa ($p = 0.8748$) was observed.

Discussion

For estimation of potential genetic risks to the germ line of human beings by environmental agents, direct studies on sperm offer an invaluable means. The use of FISH with chromosome-specific probes has opened new and attractive means to study numerical chromosomal changes in human spermatozoa. Several laboratories have published results on spermatozoa of normal men (*1–9*) and infertile men (*14,15*). Spermatozoa of translocation carriers (*16,17*), a 46,XY147,XXY individual (*18*), and an XYY individual (*4*) have also been studied with FISH.

The frequency of disomy for chromosome 1 in our study, 0.11%, fits well in the range observed in the sperm karyotype studies, i.e., 0.06 to 0.17% (*19,20*). It is also perfectly in agreement with a multiprobe FISH study on 10 normal men showing a mean chromosome 1 disomy frequency of 0.11% (range = 0.05–0.18%) (*21*). Several single-probe approaches have yielded variable results of disomy 1 in spermatozoa [for review, see (*8*)], possibly due to variable diploidy frequencies interfering with the interpretation of results. The frequency of disomy for chromosome 7 in our study (0.06%) is in the range of another study that showed 0.00 to 0.09% disomy 7 in two men (*9*).

Diploid spermatozoa may arise as a result of an error at either the first or the second meiotic division. We did not observe tetraploid sperm nuclei. Our results show rather large interindividual differences in diploidy frequencies, but the mean frequency (0.15%) is close to that reported by other groups (*3,7,14,21–23*). Nine fertile men studied using probes for chromosomes 17 and 18 showed a frequency of 0.18% diploid spermatozoa (*14*), while another study among 10 normal donors showed higher frequencies of diploidy: 0.34% by using autosomal probes and 0.45% by using sex chromosome probes (*7*). Three recent reports of studies of 10, 24, or 14 normal men of different ages showed mean frequencies of diploid spermatozoa well in agreement with our present results: 0.16%, 0.190%, and 0.145%, respectively (*21–23*). All multiprobe FISH studies indicate that diploidy is more common than disomy for a certain autosome, and thus a single-probe study cannot give accurate estimates of disomy frequencies in spermatozoa.

An increased risk of trisomy in offspring is clearly related to increased maternal age. Whether paternal age influences the risk of trisomy has been a matter of debate (*24,25*). FISH studies on human spermatozoa have opened a new way to study this question. Martin et al. (*21*) studied 10 men 21 to 52 years of age and found a significant increase of disomy 1 and YY in spermatozoa with age, but there were no effects on disomy 12, XX, or XY sperm. Griffin et al. (*22*) observed, however, that the incidence of XX, YY, and XY disomy all were significantly elevated among older men. The study consisted of 24 men 18 to 60 years of age and did not show an effect on disomy for chromosome 18 (*22*). In accordance, the study of Robbins et al. (*23*) showed significantly higher frequencies of sperm carrying sex chromosomal disomy among the older group of 4 men (mean age = 46.8 years) compared to a group of 10 younger men (mean age = 28.9 years).Together with our present results, these results suggest that the disjunction of sex chromosomes at meiotic divisions may be affected by increasing paternal age while that of autosomes may not be affected.

In conclusion, our results on the frequency of hyperploidy for chromosomes 1 and 7 in human spermatozoa in 24 normal healthy donors reveals variation between individuals. The data suggest that among spermatozoa of unexposed men diploidy is more common than disomy of a single autosome and age does not affect autosomal aneuploidy frequencies. This information is valuable for future studies on men exposed occupationally or environmentally to aneuploidogenic agents.

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